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Extracellular Matrix Induces Hormone Responsiveness and Differentiation in RUCA-I Rat Endometrial Adenocarcinoma Cells

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We recently described the establishment and the characterization of two rat endometrial adenocarcinoma cell lines which we called RUCA-I and RUCA-II. Despite fairly high estrogen receptor levels neither cell line responded to estradiol in conventional cell culture conditions on plastic and in the presence of serum. A limited hormonal response to the antiestrogen tamoxifen was detectable in RUCA-I but not in RUCA-II cells. To advance our cell culture conditions we plated RUCA-I cells on a layer of reconstituted basement membrane (Harbor Matrix) in the presence of a serum-free defined medium. These cell culture conditions induced hormone responsiveness of RUCA-I cells and permitted a stimulation of proliferation by estradiol. Further, two estradiol-induced secretory proteins with an apparent molecular weight of 115 kD and 60 kD could be identified by SDS–gelelectrophoresis if analyzed under reducing conditions. These proteins migrated as a single band in a non-reducing electrophoresis gel and were identified as components of the complement C3 system. Additionally, our results suggest that the effects of extracellular matrix and hormones on the expression of these proteins are additive. We conclude that processes of functional differentiation are most likely to occur in this *in vitro* model, particularly since the expression of components of the complement C3 system was under estrogenic control. Complement C3 proteins represent major estradiol-inducible secretory protein of the immature rat uterus *in vivo*. Culturing RUCA-I cells on top of a layer of reconstituted basement membrane provides a novel tool to study the importance of the extracellular environment on the hormone-induced gene expression in endometrial carcinogenesis *in vitro*.

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INTRODUCTION

The availability of suitable cell culture models to study hormonal aspects of endometrial cancer is very limited. Most of the published endometrial tumor cell lines of human and rodent endometrial adenocarcinoma do not express steroid hormone receptors. If they express e.g. the estrogen receptor (ER) *in vitro*, the responses to estrogens and anti-estrogens are rather marginal. Exceptions to this rule may be represented by the human endometrial adenocarcinoma cell lines Ishikawa [1] and EEC 1 [2], as well as by the rat endometrial cell lines that were obtained by retroviral mediated transfer of immortalizing and transforming oncogenes [3, 4]. In screening for suitable cell culture models, we recently described the establishment and characterization of two rat endometrial adenocarcinoma cell lines RUCA-I and RUCA-II [5]. If cultured under standard cell culture conditions, with plastic as substrate and a medium containing charcoal stripped fetal calf serum (DCC–FCS), both cell lines expressed the ER. We measured 90 fmol/mg protein in RUCA-I cells and approx. 180 fmol/mg protein in RUCA-II cells. Despite the presence of ER in both cell lines none of the cell lines responded to estradiol. The only response obtainable by hormonal treatment was a moderate

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growth inhibition of RUCA-I cells by the anti-estrogen tamoxifen. Investigating the influence of estrogens on gene expression we neither detected an upregulation of the progesterone receptor (PR), nor did hormonal treatment significantly change the expression pattern of secretory proteins [5–7]. For RUCA-I cells these findings were in contrast to the hormone responsiveness of the spontaneously developing parental endometrial adenocarcinoma of the DA/Han rat [8] and of the EnDA transplantation tumor, established thereof [9, 10].

In recent studies cell culture requirements have been elaborated for organotypic cultures of normal endometrial glands and glandular fragments in primary cell culture. Epithelial morphology of human endometrial glands was preserved much more in cultures on ECM substrates, such as matrigel^{TM} or floating collagen gels as compared to cultures grown on plastic in fetal calf serum [11–14]. Using immature rat uterine epithelial cells a morphological and functional polarity of these primary cell cultures could be demonstrated [15], with a vectorial secretion of proteoglycans [16].

This profound influence of the extracellular matrix on morphological and functional differentiation prompted us to assume that a reconstituted ECM would also provide an ideal substrate for growth and differentiation of rat endometrial tumor cells. Our working hypothesis was that ECM substrates could possibly induce functional differentiation, thereby inducing hormone responsiveness in RUCA-I cells.

In this paper we report on the effects of a reconstituted basement membrane (Harbor Matrix) from the Engelbreth Holm Swarm mouse sarcoma [17] on the morphological and functional differentiation of RUCA-I rat endometrial adenocarcinoma cells. We were particularly interested in the expression of complement C3 components, which have been described to be major estradiol inducible proteins in the uterus of immature rats *in vivo* [18, 19]. In order to simplify the system and to avoid confusing results with partial agonists we studied the effects of estradiol and the pure antiestrogen ICI 164384 [20, 21] in this experimental system.

MATERIALS AND METHODS

Cell culture

From RUCA-I cells large quantities of cells of passages 10 and 50 were grown, frozen and subsequently stored in liquid nitrogen. This enabled us to use the same stocks of cells for all experiments. Prior to experimental use, RUCA-I cells were precultured for one passage in DMEM/F12 medium without phenol red containing 10% fetal calf serum and for two passages in the above medium containing 5% dextran-coated charcoal treated fetal calf serum (DCC–FCS). Thereafter cells were harvested and 250,000–300,000 cells were seeded on top of a layer (300 μl) of ECM-substrate (Harbour matrix; Cell Systems, Germany) per well of a 24-well plate in the presence of 2 ml serum-free defined medium (SFDM). The SFDM was composed of DMEM/F12 and contained additionally 2 μg/ml insulin, 4 mM glutamine, 40 μg/ml transferrin, 10^{-8} M hydrocortisone, 2×10^{-8} M sodium selenite, and 1 μg/ml putrescine. The cells were cultured in a humidified 5% CO_2 atmosphere at 37°C and medium was changed twice a week except for Matrigel cultures, which were fed daily.

Hormonal treatment

For hormonal treatment we used estradiol (10^{-8}–10^{-6} mol/l) as agonistic ligand and as antiestrogen we used ICI 164384 (kindly provided by Dr A. E. Wakeling, Zeneca Chemicals, Macclesfied, U.K.) in concentrations from 5×10^{-8}–5×10^{-7} mol/l. This means hormonal components were used in approximately equipotent concentrations because the relative binding affinity of ICI 164384 to the ER is approx. 5-fold weaker than that of estradiol [21]. Prior to hormonal treatment cells were seeded on ECM in the above numbers and precultured in SFDM on the substrate for 24–48 h. Thereafter cells were incubated for 48 h with estradiol or ICI 164384. Control cultures received ethanol, which was used as a vehicle. Medium was changed daily and hormonal treatment was repeated. In some experiments additional controls were included by culturing RUCA-I cells in 24-well plates on plastic in the presence of DMEM/F12 medium containing 5% DCC–FCS or SFDM and the above concentrations of hormone or anti-hormone.

Metabolic labelling of secretory proteins and immunoprecipitation of complement C3 components

After 48 h of hormonal stimulation secretory proteins were labelled metabolically with [^{35}S]methionine. During labelling cells were cultured for another 16 h in the above media and under the hormonal conditions described above, including the following modifications: the content of unlabelled methionine of the medium was reduced by 90%, instead the medium was substituted with 20 μCi/ml [^{35}S]methionine. The volume of the cell culture medium per well was reduced to 150 μl. At the end of the incubation period, cell culture supernatants containing the *de novo* synthesized metabolically labelled secretory proteins were aliquoted in a 15 μl aliquot and an aliquot containing the remainder. From the 15 μl aliquot the incorporation rate of the radioactive amino acid was determined, the remainder was used for SDS–PAGE electrophoresis. Total synthesis of secretory proteins was determined in triplicate by precipitating proteins out of 3 μl cell culture supernatant with TCA.

Prior to immunoprecipitation cell culture supernatants were preabsorbed for 1 h with Sepharose 4B (Pharmacia, Freiburg, Germany). In order to immunoprecipitate complement C3 component the follow-

ing reagents were subsequently added to labelled cell culture supernatants: 100 μl of a polyclonal anti-complement C3 antiserum (SIGMA, Deissenhofen, Germany) overnight at 4°C, 5 μl of a goat anti-rabbit IgG antiserum (Dianova, Hamburg, Germany) for 1 h at 4°C, and finally with 100 μl protein-A-Sepharose overnight at 4°C (Pharmacia, Freiburg, Germany). To controls we added 10 or 50 μg of unlabelled human complement C3 (SIGMA, Deissenhofen, Germany) to prelabelled cell supernatants prior to the immunoprecipitation procedure.

Electrophoresis

Electrophoresis was performed according to standard protocols using a discontinuous system [22], reducing and non-reducing conditions and either 5, 7.5 or 10% polyacrylamide gels. Loading of the gel was performed after the amount of acid precipitable protein had been determined. For each experimental condition the same number of counts, meaning the same amount of protein bound [^{35}S]methionine was loaded on the gel. After electrophoresis the gels were fixed, incubated in En^{3}Hance (DuPont, Germany), dried and the gel bands were visualized by fluorography.

Proliferation

Cell multiplication was measured with the EZ4U test (Biomedica, Vienna, Austria) according to the manufacturers protocol. This test basically is an advanced *in vivo* MTT-test [23]. We performed this test by determining each individual experimental condition 8-fold in 96-well microtiter plates. For measurements on plastic, 1000 cells were seeded per well of the multiwell plate in the presence of 10% fetal calf serum or SFDM. The bottom of another subset of wells was covered with 30 μl of reconstituted basement membrane. In these experiments 2000 cells were seeded on top of the ECM substrate. After 1, 3 and 4 days of culture, substrates for the EZ4U-test were added and the cell proliferation was assessed by reading the extinction of the colour dye.

Expression of ER and PR

The expression of the ER, PR and of cyclophilin, which was used as constitutively expressed control gene, was assessed on the mRNA level with a rtPCR method. Reversed transcription was performed according to the manufacturers instruction using a standard kit (GIBCO, Eggenstein, Germany). The primer sequences used for amplification of the ER ssDNA were provided by Dr M. Truss (IMT, Marburg, Germany) and the sequences used for amplification of the PR and of cyclophilin were provided by Dr R. Knauthe (Schering AG, Berlin). Single stranded cDNA obtained after reverse transcription was amplified for 35 cycles [1 min, 92°C (strand separation); 1 min, 55°C (annealing); 1 min, 72°C (extension)] using the primer pairs illustrated in Table 1. To enhance sensitivity ssDNA was amplified in the presence of Digoxigenin-dUTP. 5 μl PCR product of each reaction was combined, separated in an 1% agarose gel, blotted and developed using anti-Digoxigenin antibodies (Boehringer, Mannheim) and luminescent substrates according to manufacturers instructions.

RESULTS

Morphology

RUCA-I cells grow as a monolayer of cells with a polygonal cell shape [Fig. 1(a)] if cultured on plastic in the presence of 10% fetal calf serum or 5% DCC–FCS. If RUCA-I cells are plated in a SFDM-medium they still adhere to the plastic, however their cell shape appears more rounded [Fig. 1(b)]. In contrast, if these rat endometrial tumor cells are resuspended in SFDM and plated in a totally random distribution on top of a layer of reconstituted basement membrane, they organize themselves within hours into web-like structures as shown for a low and a high cell density [Fig. 1(c, d)].

Estrogen- and progesterone receptor

The ER of cells cultured on a reconstituted basement membrane can not be quantitated by a ligand binding analysis for two reasons. The number of cells of one well of a 24-well plate is to small for a reliable ligand binding analysis. Additionally, cells do not readily come off the cell culture substrate, not even after a fairly hard proteolytic digestion. We therefore decided to analyse the expression of the ER and PR by a rtPCR

Table 1. Primer pairs for PCR-analysis. The following primer pairs were used to analyze estrogen and progesterone receptor expression by RUCA-I cells using an rtPCR approach. As a control mRNA we used cyclophilin

Gene	Primer sequences	Fragment length (bp)
Estrogen receptor	5′GGGGATGTAGTAGGTTTGTAAG	600
	5′TCCTAACTTGCTCTTGGACAGG	
Progesterone receptor	5′CATGTCAGTGGACAGATGCT	420
	5′ACTTCAGACATCATTTCCGG	
Cyclophilin	5′GGATTCATGTGCCAGGGTGG	213
	5′CACATGCTTGCCATCCAGCC	

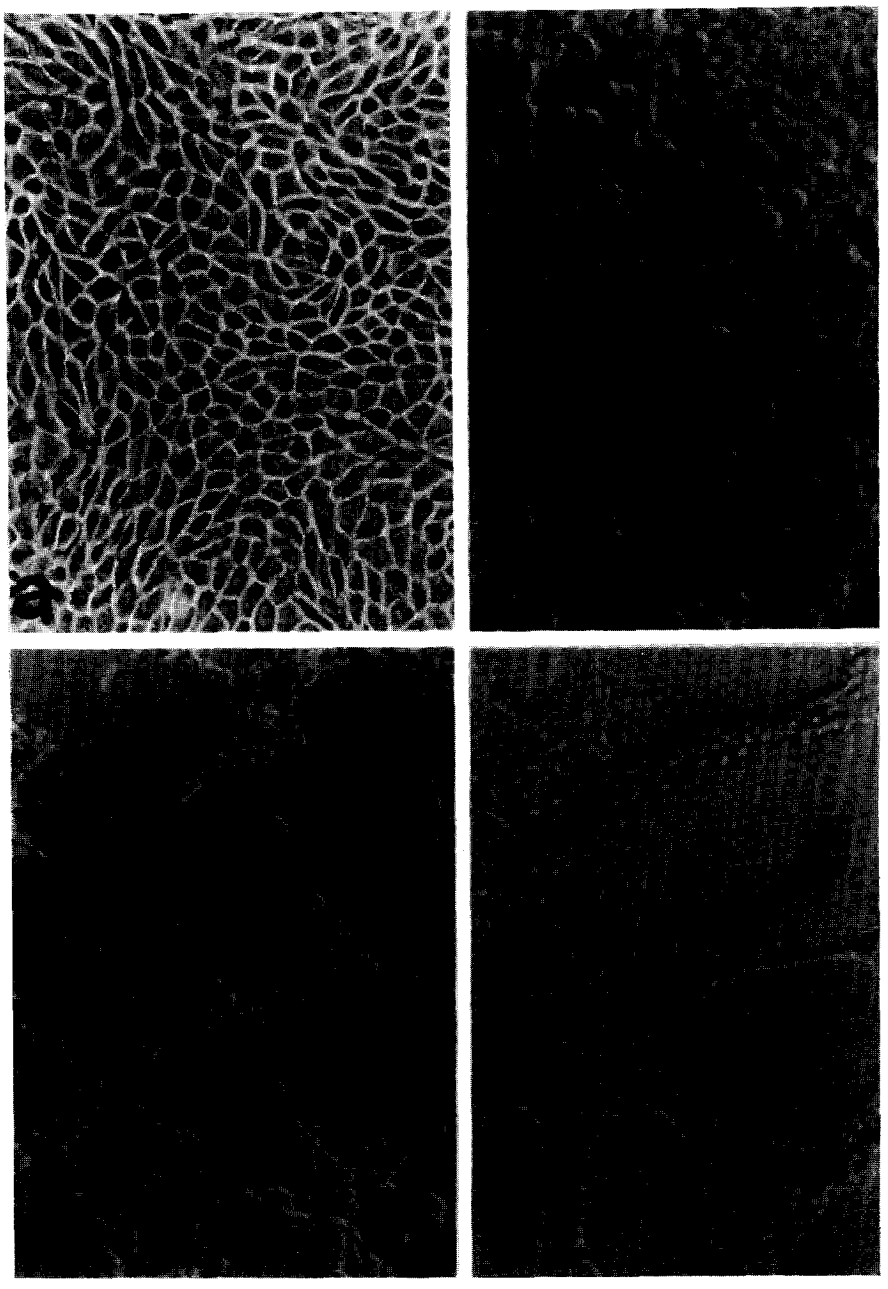

Fig. 1. The morphology of the RUCA-I cell line is dependent on the cell culture conditions. On plastic in the presence of serum RUCA-I cells grow as a monolayer with a polygonal cell shape (a), on plastic in the presence of SFDM RUCA-I cells appear more rounded (b). If cultured on top of a layer of reconstituted basement membrane they organize themselves to web-like structures as shown for low plating density (c) and a high cell density (d).

method. This method demonstrates the expression of the ER mRNA represented by an approx. 600 bp long PCR product (Fig. 2), under each experimental condition studied.

RUCA-I cells if cultured on plastic and in a DCC–FCS medium do not express the PR [5]. Here we investigated if the use of a reconstituted basement membrane in combination with a SFDM and estradiol treatment would induce PR expression. Using a rtPCR method, under neither experimental condition studied was a PR specific PCR product detectable (Fig. 2), in contrast to a mRNA originating from a normal rat uterus which was used as a positive control.

Proliferation

If RUCA-I cells were grown on top of a layer of reconstituted basement membrane, their growth velocity was reduced if compared to their growth capacity on plastic (Fig. 3). Previous findings [5] from proliferation studies with RUCA-I cells on plastic in the presence of DCC–FCS showed that the proliferation of RUCA-I cells could not be modulated by estradiol

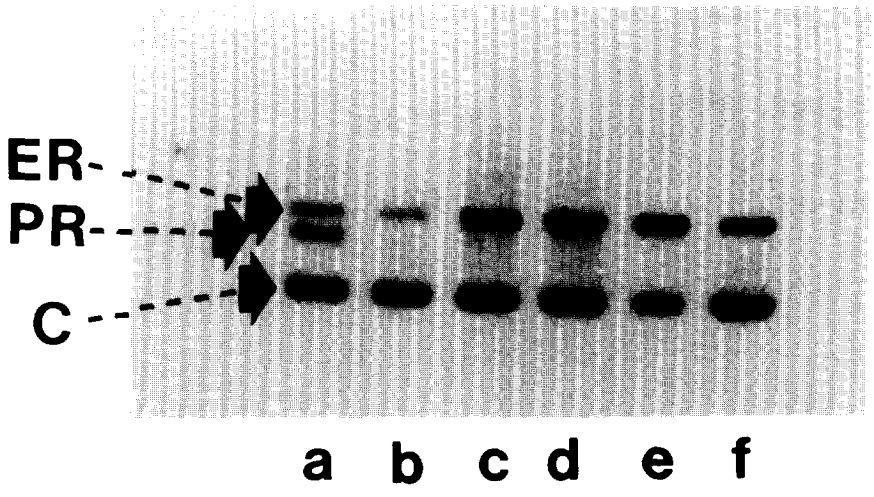

Fig. 2. Expression of the estrogen- and progesterone receptor mRNA. The ER and PR mRNA was analyzed using a rtPCR method. The ER mRNA in this analysis was represented by an approx. 600 bp fragment, the PR mRNA by a 420 bp fragment and the control gene cyclophilin by a 213 bp fragment. We analyzed mRNA from RUCA-I cells cultured on plastic in the presence of serum (b), or in the presence of SFDM (c). This 600 bp fragment was also found when RUCA-I cells were cultured on top of a layer of reconstituted basement membrane in the presence of SFDM alone (d) or after an additional treatment with estradiol (e) or ICI 164384 (f). In (g) the negative control using water instead of RNA is shown. As positive control a RNA preparation from rat uterine tissue was used (a).

treatment, but the growth velocity was reduced after tamoxifen treatment. On matrix the growth velocity of RUCA-I cells was reduced if compared to the growth velocity detectable on plastic in the presence of serum or SFDM. However, in respect to hormonal regulation of proliferation, the situation observed in cultures on a reconstituted basement membrane was completely changed. Estradiol enhanced cell proliferation, whereas treatment of cells with ICI 164384 slightly reduced the growth rate of RUCA-I cells (Fig. 3).

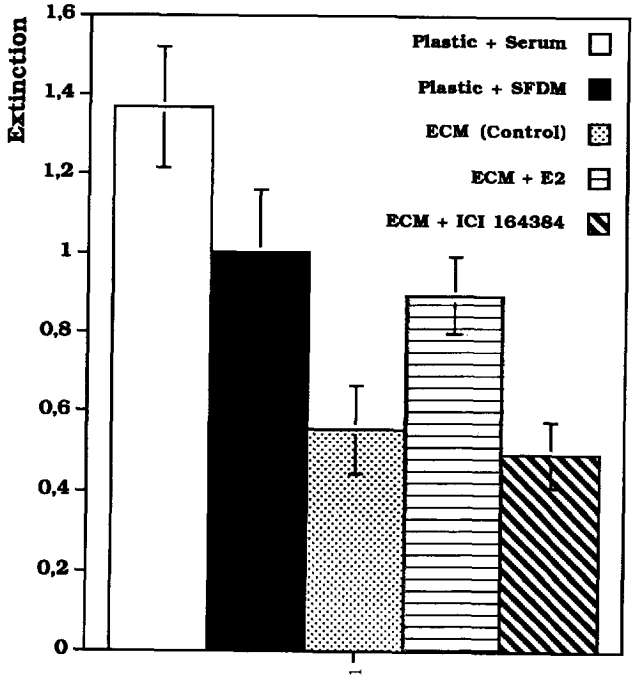

Fig. 3. Hormonal effects on proliferation of RUCA-I cells. Proliferation was measured with the EZ4U-test, which is a modified MTT-test as described in Materials and Methods. Shown is a representative experiment, given are the mean values after 4 days under the respective culture conditions in an 8-fold determination.

Hormonal effects on the expression of secreted proteins

[^{35}S]Methionine labelling of cells grown on reconstituted basement membrane was used to study *de novo* synthesis of secreted proteins. Under reducing conditions we were able to identify that estradiol at least stimulated the increased production of two secretory proteins. These estrogen regulated proteins were approx. 115 and 60 kD in size [Fig. 4(a–c)]. If the same proteins were analyzed under non-reducing conditions, it turned out, that the estradiol-induced proteins migrated as a single band of approx. 180 kD [Fig. 4(d–f)]. Occasionally, and dependent on the resolution of the gel, we additionally observed other estrogen regulated proteins with apparent molecular weights of 41, 53 and 200 kD (not shown).

Twelve experiments performed under reducing conditions, originating from two independent investigators, and five experiments analyzing non-reduced proteins were subjected to semiquantitative densitometry. This densitometric analysis revealed that the inhibiting effect obtained after treatment of cells with ICI 164384 usually reduced the production of the 115 and 60 kD protein below the unstimulated level prior to estradiol treatment (Fig. 5).

The reconstituted basement membrane promoted not only hormone sensitivity, but also completely altered the pattern of expressed secretory proteins

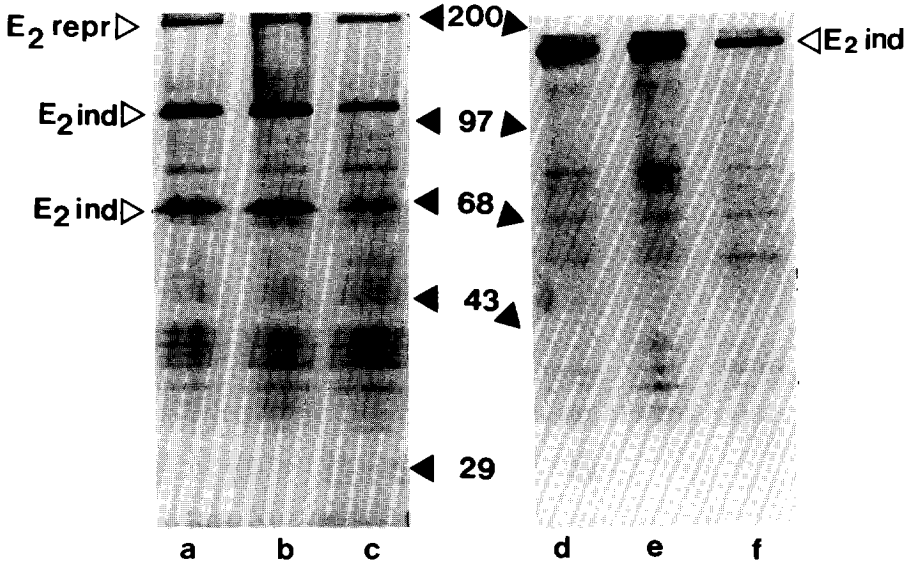

Fig. 4. Hormonal effects on secreted proteins: SDS–PAGE. RUCA-I cells were grown on a layer of reconstituted basement membrane, hormonally treated and metabolically labelled as described in Materials and Methods. Cell culture supernatants were adjusted according to the dpm measured in the acid precipitable material accumulated. Equal numbers of dpm for each experimental condition were layered on top of a discontinuous SDS–Gel and separated under reducing (left panel) or non-reducing (right panel) conditions. Cell culture supernatants from untreated controls (a, d), from estradiol treated cells (b, e) and from cells treated with ICI 164384 (c, f) are shown.

(Fig. 6). Surprisingly the pattern of expressed secretory proteins were rather similar, but not identical if *de novo* synthesized secretory proteins of RUCA-I cells grown on plastic in the presence of serum were compared to the *de novo* synthesized proteins from RUCA-I cells grown on a reconstituted basement membrane in the presence of SFDM. Reconstituted basement membrane and serum provide a rich source for adhesion factors, this may be the reason why RUCA-I cells grown in SFDM on plastic express other proteins than RUCA-I cells grown under the other two cell culture conditions and showed by far the highest number of *de novo* synthesized secretory proteins (Fig. 6).

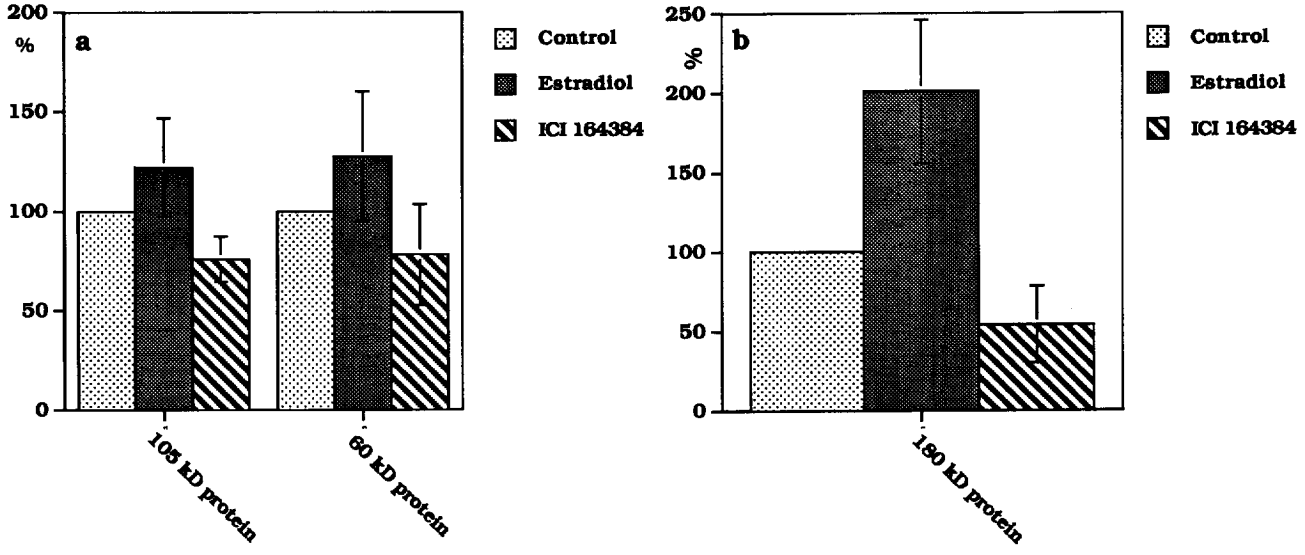

Fig. 5. Densitometry of estradiol-induced and the estradiol repressed proteins. Twelve experiments run under reducing conditions from two independent investigators and five experiments under non-reducing conditions were semiquantitatively evaluated by densitometry. The quantitative data for the 105 kD protein and the 60 kD protein (a) are shown for the reducing conditions. For the non-reducing approach the 180 kD protein (b) was evaluated. Densitometric readings of controls were set to 100% and hormonally treated groups were calculated accordingly. Shown are mean values and standard deviations.

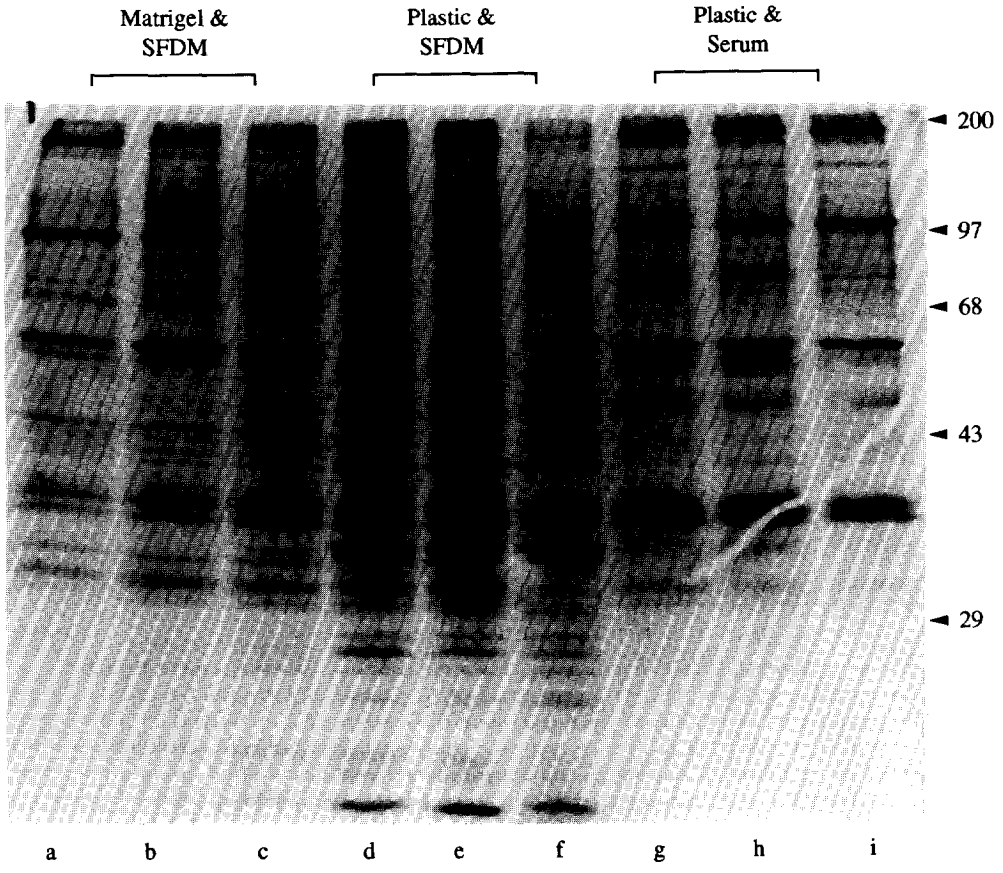

Fig. 6. Influence of cell culture conditions on the *de novo* synthesis and secretion of proteins. The pattern of secreted proteins of RUCA-I cells was analyzed in three different cell culture conditions. Condition one was reconstituted basement membrane in combination with SFDM (a–c), condition two was cell culture plastic and a SFDM (d–f), condition three was cell culture plastic in combination with medium containing serum (g–i). Additionally some cultures were treated with estradiol 10^{-8} mol/l (b, e, h), with ICI 164384 5×10^{-8} mol/l (c, f, i) or with ethanol as control (a, d, g).

To test the specificity of the estrogenic and antiestrogenic effects on RUCA-I cells, we performed competition experiments. Figure 7 shows a representative competition experiment using estradiol [Fig. 7(b)] and ICI 164384 [Fig. 7(c)] alone at equipotent concentrations of 10^{-7} or 5×10^{-7} mol/l respectively or in combination. In the experiment shown (upper panel) it becomes clearly visible that estradiol in increasing concentrations [Fig. 7(d, e)] is capable of overcoming the inhibiting effect of ICI 164384 on the expression of the 115 and 60 kD protein. This figure also shows that ICI 164384 at equipotent concentrations [Fig. 7(d)] reduces the estradiol induced production of these proteins. A concentration dependency with ICI 164384 could not be performed because this antiestrogen in our hands was not soluble at a 5×10^{-6} mol/l concentration.

As demonstrated above and in previous studies [5] we did not see any effect of estradiol treatment on RUCA-I cells, if these cells were cultured on plastic in the presence of serum. In comparison to the effects seen on an ECM substrate in the presence of SFDM the question arose, could hormonal effects be detectable in an experiment with RUCA-I cells plated on a reconstituted basement in the presence of a medium containing 5% DCC–FCS. Under both serum-containing and serum-free conditions, the 115 and the 60 kD protein were expressed, however, serum inhibited their hormonal regulation. In experiments directly comparing these two cell culture conditions we found an estradiol-induced production of the 115 kD protein and the 60 kD protein only in combination with SFDM [Fig. 8(e)] but not in the presence of charcoal stripped fetal calf serum [Fig. 8(b, c)]. This means that serum factors possibly suppress hormone responsiveness of RUCA-I cells.

Identification of the estradiol-inducible proteins

Components of the complement C3 systems were identified as major estradiol-inducible, secretory proteins in the uterus of immature rats *in vivo* [18, 19]. Since the estimated molecular weights of the estradiol-inducible proteins of RUCA-I cells approximately matched the size of rat complement C3 components and particularly since both proteins migrated as a single band under non-reducing conditions, we immunoprecipitated [^{35}S]methionine labelled cell culture supernatants with anti-complement C3 antibodies. The 115

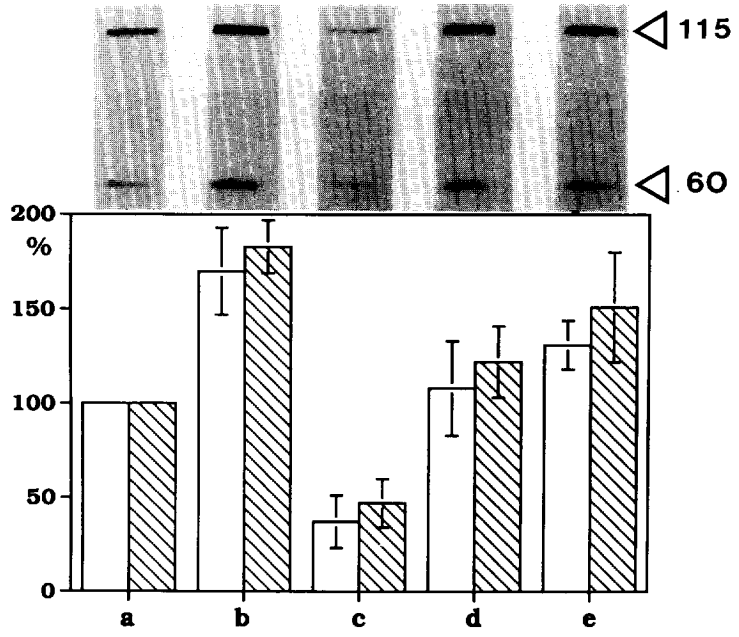

Fig. 7. Competition experiments: RUCA-I cells were grown on a reconstituted basement membrane in SFDM either without hormonal treatment (a), in the presence of 10^{-7} mol/l of estradiol (b), 5×10^{-7} mol/l of ICI 164384 (c) or equipotent concentrations of both hormone derivatives (d), as well as in a 10-fold excess of estradiol (5×10^{-6} mol/l) over ICI 164384. The upper panel shows representative experiments, the lower panel shows mean values and standard deviations of the densitometric evaluation of all competition experiments ($n = 4$). Values obtained for the untreated controls were set to 100% and all other values calculated accordingly. Open columns represent the 115 kD protein, striped columns represent the 60 kD protein.

and 60 kD proteins were precipitated [Fig. 9(a)]. Specificity of the reaction was verified by competing the radiolabelled precipitate with 10 μg and 50 μg of unlabelled complement C3 [Fig. 9(b, c)].

DISCUSSION

With this paper we demonstrated that a reconstituted basement membrane in combination with SFDM induces hormone responsiveness and differentiation in cultured RUCA-I rat endometrial adenocarcinoma cells. We detected estrogen-regulated secretory proteins and identified them as components of the complement C3 system. Importantly, treatment with the antiestrogen ICI 164384 suppressed the formation of complement C3 components in RUCA-I cells below the unstimulated level. The latter finding suggests an additive effect of estradiol and reconstituted basement membrane on gene expression of hormone dependent genes, with both effects being inhibited by the anti-estrogen treatment. To our knowledge, we describe for the first time functional differentiation of an endometrial tumor cell line *in vitro* with estrogenic effects on gene expression. The RUCA-I cell line and the hormone inducibility of gene expression represent a unique tool to study agonistic and antagonistic functions of estrogens and anti-estrogens in endometrial carcinogenesis *in vitro*. We observed an increased expression of a 115 kD protein and a 60 kD protein, which migrated as a single band in non-reducing gels, if RUCA-I cells were treated with estradiol. This induction could be suppressed below the values of the unstimulated control by treatment with the anti-estrogen ICI 164384. Occasionally and dependent on the resolution of the polyacrylamide gel, we additionally observed a 40 kD protein to be inducible by estradiol and 53 kD and 200 kD proteins to be repressed in the presence of estradiol.

Out of these observations two points deserve extensive discussion: the molecular nature of the induced proteins, and the issue of suppression by the anti-estrogen ICI 164384 ending far below basal, unstimulated values. Sundstrom *et al.* [18] described the estrogenic regulation of the tissue specific expression of the complement C3 in the immature rat uterus *in vivo*. The complement C3 was thereby produced and secreted by the glandular and the luminal epithelium only. The molecular weight of the components of the rat complement were assessed as 110 and 74 kD [19] or 115 and 65 kD [18]. These apparent molecular weights described for rat complement C3 components do not completely match the molecular weights of the proteins, which were upregulated after estradiol treatment in our experimental system, which were calculated as 115 and 60 kD, however they are in a similar range. Additionally, the components of the complement C3 are not only rather similar in size to those proteins being inducible in our experimental system but behaved in a manner biochemically very similar to the proteins regulated by estradiol in RUCA-I cells.

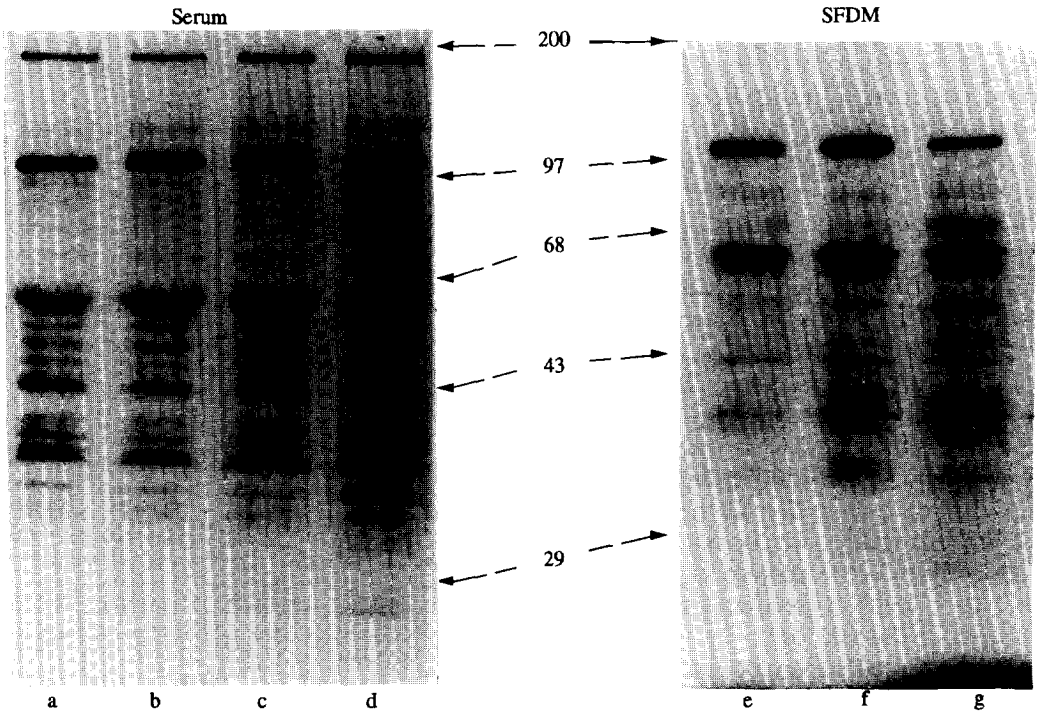

Fig. 8. Comparison of serum-containing and serum-free cell culture media. The effect of serum on the hormone dependent secretion of the 105 and 60 kD protein on top of reconstituted basement membrane was analyzed. Cells were cultured on a reconstituted basal membrane in the presence of serum containing medium (a–d) or SFDM (e–g). Untreated controls are shown in (a) and (e), estradiol treatment 10^{-8} mol/l (b), (f) or 10^{-7} mol/l (c) and treatment with 5×10^{-8} mol/l of ICI 164384 (d), (g) are shown in the other lanes.

Components of the C3 complex were found to be induced by both estrogens and most of the anti-estrogens. The only anti-estrogen that suppressed their expression was ICI 164384 [24, 25]. Preliminary results of our own laboratory suggest an increased formation of the 115 and 60 kD proteins from RUCA-I cells after treatment of cells with the anti-estrogens Tamoxifen and ZK 119010 (Vollmer unpublished observations). The only substance with almost complete inhibitory activity on RUCA-I cells was the pure anti-estrogen ICI 164384. Further, complement C3 components of the immature rat uterus migrated as a single band under non-reduced conditions, as did the stimulated proteins in RUCA-I cells. Finally, by immunoprecipitation we presented direct evidence that the stimulated secreted proteins of RUCA-I cells are indeed components of the complement C3 complex. In summary, if RUCA-I cells are cultured on a reconstituted basement membrane, expression of complement C3 is under control of estradiol, like it is *in vivo*. It is remarkable that the hormonal regulation of these proteins could not even have been mimicked *in vitro* in highly elaborative culture conditions for normal rat uterine epithelium under polarized conditions. In this approach complement C3 components were expressed, but were not under the control of estradiol [26].

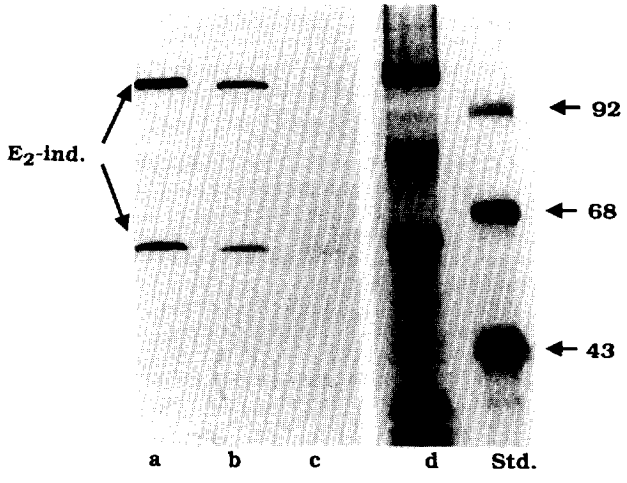

Fig. 9. Immunoprecipitation of complement C3. The identity of the estradiol-induced proteins was examined. For this purpose [35S]-methionine labelled cell culture supernatants were immunoprecipitated with anti-complement C3 antibodies. The precipitation in the absence (a) and presence of 10 μg (b) or 50 μg (c) unlabelled complement C3 added to the supernatants is shown in comparison to the untreated cell culture supernatants (d) and a molecular weight standard.

The complexity of effects seen after a treatment with ICI 164384 alone also was very surprising. Most intriguing was the fact that ICI 164384 if given alone, suppressed expression and formation of the 115 and 60 kD protein significantly below the unstimulated (control) levels. This finding suggests the hypothesis that the effects of a reconstituted basement membrane and a hormonal treatment on gene expression in RUCA-I cells are additive in regard of the formation

and secretion of the proteins described above. A possible mechanistic explanation for this effect could be deduced from recent data on ligand-independent activation of steroid hormone receptors. It has been shown that activated phosphorylation pathways, e.g. by dopaminergic receptors [27, 28] or simply by stimulating cultured cells with agents that increase intracellular phosphorylation levels, e.g. 8-bromo-cAMP or okadaic acid [29], activated steroid hormone receptors independent of the ligand. Since integrin-mediated attachment of cells to extracellular substrates can activate various intracellular second messenger systems, among them some that are coupled to phosphorylation pathways, it could well be that this integrin/ECM-interaction leads to phosphorylation and ligand-independent activation of the ER [30–32]. This assumption further implies an inhibition of both estrogenic and ligand-independent activation of the ER by antiestrogens as has already been demonstrated for EGF-dependent [33] and dopaminergic activation of the ER [34].

Finally, the reconstituted basement membrane itself may not directly induce hormone responsiveness of RUCA-I cells, instead the ECM substrate may induce functional differentiation in tumor cells, as it does in a variety of glandular epithelial cells including epithelial cells of the mammary gland [35–41] and the endometrium [11–14]. As a secondary response to differentiation processes RUCA-I cells may acquire hormonal responsiveness. Which pieces of evidence have been observed that may support the hypothesis of a functional differentiation of RUCA-I cells if cultured on top of a layer of reconstituted basal membrane? First, on ECM substrate RUCA-I cells do not grow randomly distributed, but instead assemble themselves to highly organized 3-dimensional, net-like structures. Second, on reconstituted basement membrane, proliferation was slowed down if compared to cultures of RUCA-I cells on plastic substrates. Third, on top of a reconstituted basement membrane RUCA-I cells acquired estradiol responsiveness of complement C3 proteins, therefore the *in vitro* situation matched the observations made *in vivo*. These three pieces of evidence support the hypothesis that processes of functional differentiation occur in RUCA-I cells if they are cultured on ECM substrates.

In conclusion, we presented a novel, hormone responsive endometrial tumor cell line. We described the cell culture requirements for the induction of hormone responsiveness in these rat endometrial adenocarcinoma cells: that is a reconstituted basal membrane in combination with SFDM. Further our results suggest an additive effect of ECM-substrate and hormonal treatment on gene expression. All taken together, the presented cell culture model appears to be a potent tool to study functions of estrogens in endometrial carcinogenesis *in vitro* and to study possible prospects of anti-estrogens in the treatment of endometrial cancer. Finally, this cell culture model provides the basis to isolate and characterize hormone-induced and hormone-repressed genes in endometrial cancer.

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